Microfibrillar meshwork of the synovial lining and associated broad banded collagen: a clue to identity

J R Levick, J N McDonald

Abstract

The surface layer of synovial interstitium lining the rabbit knee was studied by transmission electron microscopy. Over a distance of 2–3 μm normal to the surface the interstitium contained a network of fine microfibrils (diameter 9.3 (0.7) nm, mean (SEM)) which was quite dense in places (fractional area of projection 0.189 (0.023)), and stained with ruthenium red. Periodic collagen fibrils were relatively scanty and fine (diameter 32 (2) nm) in this surface layer. Broad cross-striated bundles occurred in association with the microfibrils and B cells. These fibrous long spacing bundles (FLS) had a single period of 92.8 (2.8) nm with a broad dark band (37.6) (1.8) nm—so called 'zebra collagen'. Both the periodicity of the FLS and the morphological characteristics of the microfibrils are typical of type VI collagen, a widespread constituent of soft connective tissues. The functional importance of the inner microfibril network is likely to be mechanical, biochemical (glycosaminoglycan and glycoprotein entrapment), and to a very minor degree hydraulic resistance.

The interstitium of the synovial lining is a complex matrix which, together with the capillary wall, governs the movement of fluid, nutrients, and proteins between plasma and the joint cavity.¹ It also governs the clearance of intra-articular proteins, proteoglycan fragments, and immune complexes into lymph. Thus the nature of the matrix influences the intra-articular environment. Despite this the composition of the matrix remains poorly defined. The major identified constituents are structural collagen fibrils,^{2–3} the structural glycoprotein fibronectin,^{3–4} and glycosaminoglycans, probably mainly as proteoglycans.^{5–9} The classic structural elements are the periodic collagen fibrils of diameter 50 nm or more, which abound in the deeper zones of the synovial lining (>2 μm) and are composed of type I and type III collagen molecules (Ashhurst and Levick, unpublished data).^{3–10} There is also a network of finer non-periodic structural filaments located predominantly in the zone of interstitium adjacent to the joint cavity. These synovial 'microfibrils' were discovered a considerable time ago by Highton, Myers, and Rayns⁸ but have received comparatively little study. We report here some morphological clues that synovial microfibrils may be related to type VI collagen, and assess the potential functional significance of the microfibrils. The type VI collagen molecule was

characterised quite recently.^{11–16} It is widely distributed in soft connective tissues,¹¹ intervertebral disc,¹² articular cartilage,¹³ and fibroblast culture matrix¹⁴ but has not been sought in synovium. Because of its short triple helix and large terminal globular domains the 100 nm long molecule readily associates into tetramers, which assemble end to end into microfibrils. It can also form broader, banded aggregates called fibrous long spacing material (FLS) with a characteristic single period of ~ 100 nm in electron micrographs.^{15–16} The broad stripes of this aggregate have earned it the appropriate name of 'zebra collagen'. Fibrous long spacing material can be produced artificially in vitro from type I collagen molecule too, but a mixture of FLS periods results, mainly 200–300 nm (FLS types I, II, and IV) or occasionally 90 nm (FLS III).^{17–20} The existence of FLS in synovium was noted by two groups^{9–21} before the characterisation of type VI collagen. We have therefore re-examined the morphology of synovial FLS.

Materials and methods

Synovial samples were taken immediately after death from knees of New Zealand albino rabbits weighing 2–3 kg. Samples of size ~ 5 mm $\times$ 3 mm $\times$ 1 mm thick were excised from the lateral or medial suprapatellar pouch, the infrapatellar fat pad, and the posteromedial pouch. The tissue was harvested under a variety of conditions: from fresh joints without any previous intervention ($n=5$); from joints fixed in situ by intra-articular infusion of half strength Karnovsky fixative under 5 cmH₂O pressure²² ($n=5$) or 25 cmH₂O ($n=5$); and from joints which had been subjected to saline infusions in the course of experiments to record trans-synovial flows²³ ($n=2$). The nature of the microfibrils and FLS did not seem to vary under these different conditions, and we therefore present the observations as a single group.

Tissue was fixed for two hours in half strength Karnovsky solution (2% paraformaldehyde, 2.5% glutaraldehyde in 0.05 M cacodylate buffer), washed in buffer, diced finely, and then fixed in 1% osmium tetroxide. Ruthenium red, a cationic inorganic complex, was added to both fixative and buffer in a concentration of 1 g/l for some samples in order to stain interstitial anionic components. After dehydration through an ascending series of ethanols the tissue was embedded in low viscosity resin (Agar Aids, Cambridge) at 60°C. Linear macroscopic shrinkage of the samples was 4–8%. The observed mean collagen repeat distance of 59.7 nm (see text), compared with

Department of
Physiology,
St George's Hospital
Medical School,
London SW17 0RE
J R Levick
J N McDonald

Correspondence to:
Dr Levick.

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Figure 1: Microfibrillar network of the synovial lining in the rabbit knee (suprapatellar pouch). (a) Synovium showing predominantly superficial microfibrils and predominantly deeper classic collagen fibrils. Arrows point to small fibrous long spacing bodies. J=joint cavity. Conventional heavy metal staining. Joint had been subjected to trans-synovial flow of mammalian Ringer solution before sampling. Bar=500 nm. (b) Higher power of microfibrils (short arrows) abutting directly on to the joint cavity (J). Cf is a major collagen fibril. Areolar muscular synovium excised from knee of normal, freshly killed rabbit and stained with ruthenium red. Bar=100 nm.

an accepted biochemical periodicity of 64–67 nm, indicated an average 7–11% shrinkage of striated collagen fibrils.

Embedded tissue was oriented under a binocular microscope and sectioned normal to the synovial surface on a Reichart-Jung microtome. The section interference colour was silver, indicating a section thickness of the order 80 nm. Sections were stained conventionally with uranyl acetate and lead citrate²⁴ and examined in a Phillips 201 electron microscope at 80 kV. The instrument's magnification was calibrated regularly using a diffraction grating of 2160 lines/mm, with a stated accuracy of $\pm 1\%$.

Results

DISTRIBUTION OF FIBRILS AND MICROFIBRILS

Figure 1a illustrates the general distribution of microfibrils and classic collagen fibrils in the surface zone of the synovial lining. Fine microfibrils abound in the interstitium within a distance of 2–3 μm normal to the joint cavity and form a meshwork, whereas periodic collagen fibrils are scanty in this zone. The microfibrillar network abutts directly on to the synovial fluid (fig 1b), whereas collagen fibrils rarely do so. Beyond 2–3 μm from the joint cavity the compositional balance reverses, with coarser periodic collagen fibrils in bundles

predominating, though microfibrils still occur and in places interpenetrate the collagen bundles (fig 1a). Although the microfibrils often give the appearance of branching, this appearance could simply be due to overlap of the narrow microfibrils within the 80 nm thick sections. Although microfibrillar regions were readily found, there were also some superficial regions where the matrix was more granular or amorphous, perhaps because the section was perpendicular to the predominant lie of the microfibrils.

Figure 2: High magnification of interstitium in normal areolar-muscular synovium from untreated rabbit suprapatellar pouch, stained by ruthenium red. The arrowed microfibril is 1.8 μm from the synovial surface and shows evidence of internal structure. cf=collagen fibril. Bar=100 nm.

DIMENSIONS AND DENSITY

The diameter of the microfibrils averaged 9.3 (0.7) nm (mean (SEM)) for 15 sharply defined examples (range 5.5–13.7). This fulfils the criteria of Cleary and Gibson, who define a microfibril as any filament of diameter <20 nm and >5 nm which lacks the 67 nm periodic banding of collagen fibrils.²⁵ Similar non-banded microfibrils in human synovium⁸ and mouse²¹ have reported diameters of 17–25 nm and 10 nm respectively. The periodic collagen fibrils were of much greater diameter, averaging 50 (3) nm (mean (SEM)) in 42 collagen bundles. The superficial periodic collagen fibrils were scattered rather than in bundles and were noticeably smaller, mean diameter 32 (2) nm ($n=12$; $p<0.01$, t test). The maximum uninterrupted lengths of observed microfibrils were 500–1000 nm, but most were considerably shorter.

The density of the superficial microfibrillar meshwork may affect hydraulic resistance (see 'Discussion'). As an index of density, the fraction of interstitial space apparently occupied by microfibrils was estimated by a standard point-counting method—namely, the number of superimposed grid points overlying microfibrils relative to total grid points.²⁶ In five samples where microfibril resolution was optimal and the lie of the microfibrils was apparently longitudinal the microfibril profiles occupied 18.9 (2.3)% (mean (SEM)) of the interstitial area. Since section thickness (t) was nearly $10\times$ the microfibrillar diameter (d) this must be allowed for in evaluating microfibril volume density. If the microfibrils are aligned perfectly parallel to the cut plane, and overlap is negligible, simple geometry shows that the microfibril volume fraction equals apparent area fraction times $\pi d/4t$. If the microfibril orientation is completely random the factor becomes $\pi d/2t$. If 80 nm is adopted as the typical thickness of a silver section the proportion of interstitial volume occupied by microfibrils is therefore between 1.73% and 3.46%.

The microfibrils stained positively with ruthenium red (figs 1b and 2) but were also distinguishable, albeit somewhat pale, after conventional heavy metal stain alone (fig 1a). Under high magnification there was evidence of internal structure. The resolution of this structure was not very good, but at favourable sites there seemed to be globular domains (~ 14 nm diameter) joined by finer linear elements (fig 2).

FIBROUS LONG SPACING BODIES

Although not present in every field, these striking structures were quite common. They comprised wide elongated bodies with a regular dark broad transverse stripe (figs 3 and 4). They were found in association with the microfibril meshwork and in some cases in close association with the fibroblast-like B cell (fig 3). Fibrous long spacing bodies were up to 5000 nm long (though typically only 300–500 nm) and 100–300 nm broad. The transverse striations had a periodicity of 92.8 (2.8) nm (mean (SEM)) of 20 FLS, range 77–132 nm). The dark bands were on average 37.6 (1.8) nm wide (mean (SEM); $n=20$) and their appearance was quite

Figure 3: Synovium from posterior pouch of an untreated rabbit knee. A fibrous long spacing bundle (FLS) (arrow) is closely associated with a fibroblast-like B cell. At higher magnification the FLS dark bands can be resolved into dark globules in lateral register. The pale bands are traversed by very fine lines. Conventional heavy metal stain. J=joint cavity. Bar=300 nm.

Figure 4: Fibrous long spacing bundle in interstitium of posteromedial pouch synovium, from a rabbit knee fixed in situ by intra-articular fixative at 5 cmH₂O pressure. Conventional heavy metal staining. Bar=200 nm.

Figure 5: Small fibrous long spacing bodies (broad arrow) in infrapatellar adipose synovium of untreated rabbit knee, showing characteristic association with a collagen fibril. The tissue was stained with ruthenium red, which decorates the classic collagen fibril in regular protruding rings (arrow heads). Bar=200 nm.

characteristic, with individual units often discernible within the band, and often with a slight 'stagger' in the transverse register. The periodicity of mouse synovial FLS is reported to be similar, 90–120 nm,^{9 21} as is the distribution.²¹ Under high magnification it was just possible to discern fine linear structures traversing the pale band and connecting the dark bands (fig 4), as in type VI collagen FLS. Unlike the FLS formed by type I collagen, the synovial FLS had no intraperiod banding. Also, whereas several forms of FLS (I–IV) appear as a mixture in a single preparation of FLS from type I collagen,¹⁸ only a single form of FLS was observed in synovial interstitium.

Another characteristic of FLS was its tendency to occur alongside an isolated periodic collagen fibril, running parallel to the fibril but not in

periodic register. In fig 5, which shows a ruthenium red stained example, the repeat length of the FLS is clearly different from that of the collagen fibril. The mean period of the collagen fibrils was 59.7 (2.3) nm (n=5). Like Highton *et al*⁸ and others^{13 27} we noted that ruthenium red studded the collagen fibril at regular intervals, indicating a periodic distribution of collagen bound anionic material.²⁸

Discussion

The 2–3 µm of synovial matrix adjacent to the joint cavity contains three supramolecular assemblies: periodic collagen fibrils (scattered), non-periodic microfibrils (abundant), and fibrous long spacing fibres (occasional). Previous observers of the last two elements, before the

biochemical characterisation of type VI collagen, suggested various compositions: acid mucopolysaccharides,⁸ collagen-glycosaminoglycan complexes,²¹ or collagen affected by collagenase.⁹ Okada *et al* noted that the microfibrils and FLS of mouse synovium were resistant to 20 minutes' digestion by α -chymotrypsin, trypsin, papain, and chondroitinase ABC.⁹ In the light of recent work characterising the morphology of type VI collagen *in situ*^{11–16} we suggest that the FLS is composed of type VI collagen, and that the associated microfibrils too may contain type VI collagen. The features which indicate this are as follows:

(1) Type VI collagen is extremely widespread in soft connective tissues—placenta, blood vessels, skin, kidney, liver, muscle, tendon, uterus, corneal stroma, annulus fibrosus, nucleus pulposus, femoral head cartilage, perichondrium, chick embryo, and fibroblast cultures. Type VI collagen is present 'in most of the extracellular spaces which also contain collagen I and III and fibronectin'.²⁹ As synovium is a relatively unspecialised soft connective tissue, whose cell population is 67% fibroblast-like B cells and whose interstitium contains types I and III collagen and fibronectin,³ the presence of type VI collagen seems likely *a priori*.

(2) Undegraded type VI collagen exists *in situ* as microfibrils in placenta and aorta, as a fine granular meshwork in cross section, and as more amorphous regions.¹¹ The diameter of synovial microfibrils (mean 9.3 nm, range 5.5–13.7) fits well with the diameter of proved type VI microfibrils (5–10 nm).¹¹ Published electron micrographs of proved type VI microfibrillar networks in other tissues closely resemble the synovial meshwork (figs 1 and 2) in general pattern, lengths, and densities—see for example figs 8 and 9 in reference 11.

(3) Type VI collagen molecules form tetramers, in which globular domains are linked by finer strands.^{12 15 16} Although internal microfibril structure was not well resolved here, globular elements linked by finer strands were occasionally detected (fig 2).

(4) Type VI collagen often aggregates into FLS, with a single repeat period of 90–120 nm due to a single repeating broad dark band, which gives rise to the name zebra collagen.^{15 16} The single repeat period for synovial FLS was 92.8 (2.8) nm and the resemblance to published electron micrographs of type VI FLS—for example, fig 5 of reference 16 and fig 9 of reference 13—is most striking. In contrast, the FLS formed *in vitro* by precipitation of the classic collagen, type I, has mostly periods of 200–300 nm (FLS types I, II, and IV). A form with a major period of 90 nm is also known (FLS type III); but its appearance is quite unlike that of type VI FLS or synovial interstitial FLS in that it has many fine intervening dark bands per major period, rather like its relatives the classic collagen fibrils.^{17–19} It is not clear whether type VI FLS exists naturally *in vivo* or is formed by microfibrillar aggregation during processing.

(5) The width of the synovial FLS dark band (37.6 (1.8) nm) is not far removed from that of type VI globule-pairs (43.5 nm)¹⁵; and the paler

interspaces (55.2 nm) are not far removed from the lengths of type VI thin segments (58–66 nm).^{12 15} The existence of fine filamentous strands traversing the pale zones (triple helix regions of tetramers) is characteristic of type VI collagen FLS and was detected in synovial FLS. The detection of individual domains within the dark band, and the tendency of their transverse register to stagger is another shared characteristic.

(6) Type VI microfibrils and type VI FLS dark bands stain with ruthenium red and more faintly with heavy metals,¹³ as do the synovial analogues.

The similarities therefore seem strong enough to indicate that synovial FLS is composed of type VI collagen; and this raises the possibility that the associated synovial microfibrils too may contain type VI collagen. Proof of the latter suggestion will require immunocytochemistry and immunoelectron microscopy as both type V collagen³⁰ and the recently described 'fibrillin' can also form microfibrils—though the former are associated with non-capillary basement membranes (lacking in synovium) and the latter has a 67 nm period and is associated with elastin.³¹ Immunohistochemical studies are at present prevented by the unavailability of an anti-type VI antibody active against rabbit type VI collagen, because such antibodies are mostly raised in rabbits. Microfibrils occur in human synovium too,⁸ as does FLS.²¹ Preliminary electrophoretic analyses of samples of synovial lining tissue taken from rabbit and human knee joints confirm that type VI collagen does exist in the lining (Eyre, Wu, and Simkin, personal communication). This obviously raises questions about the antigenicity of the microfibril network in rheumatoid disease.

We considered three functions for the microfibrillar network which do not depend on its exact chemical identity.

(1) Preservation of the glycosaminoglycan and glycoprotein content of the synovial lining in the face of trans-synovial flow requires that some fixed structural scaffolding exists. In articular cartilage, for example, the proteoglycan is retained ultimately by the type II collagen network. There are very few periodic (type I/III) collagen fibrils in the innermost synovial matrix, however. The fine, dense meshwork of microfibrils seems well suited for a role as an 'anchor' for molecular fibres, whether by chemical bonding or simple physical entanglement.

(2) In view of the paucity of type I/III fibrils in the innermost synovial lining the mechanical properties of the microfibrillar network may contribute to the lining's extensibility and tensile properties.

(3) The projection of the microfibrillar network on the observed plane is quite dense, which raises the possibility that it may contribute significantly to the substantial hydraulic resistance of synovial interstitium. The area density is, however, to some extent an illusion created by the finite section thickness, and the volume density is only 1.7–3.4%. Nevertheless, cylindrical microfibrils of radius 4.65×10^{-7} cm (r) occupying 0.0173–0.0346 of the interstitial

volume (ϕ) have an enormous surface area ($S=2\phi/r$)—namely, 7.4×10^4 – 14.9×10^4 cm²/cm³ interstitium (similar to the surface area of classic periodic collagen fibrils in the deeper layers of articular cartilage or proteoglycan in scleral stroma³²). The ‘mean hydraulic radius’ within such a network is $(1-\phi)/S$, or 65–132 nm (for theory see reference 32). The large surface area and small hydraulic radius create hydraulic drag—that is, resistance to fluid exchange. For a void volume of 0.983 ($1-\phi$) and a Kozeny factor of 36.3 the resistance is 1.5×10^9 dyne.s.cm⁻⁴.³² This is less than 1% of the synovial interstitial resistance (2×10^{11} – 5×10^{11} dyne.s.cm⁻⁴).^{32, 33} The microfibril meshwork of the synovial lining is thus unlikely to be of direct hydraulic significance but may have as its major role the entrapment of the molecular fibres—for example, proteoglycans—which themselves dominate hydraulic resistance in most tissues.³²

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